

Neurotrophic Factor Secretion and Neural Differentiation Potential of

Multilineage-differentiating Stress-enduring (Muse) Cells derived from Mouse Adipose Tissue

(マウス脂肪組織由来 Muse 細胞の神経栄養因子と神経分化能についての検討)

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## **Abstract**

It has been reported that female rats have a sex-related advantage in functional recovery and neuroprotection after spinal cord injury (SCI). However, the association between anxiety and neurological function after SCI in female and male rats remains unclear. The purpose of this study was to examine sex-related differences in anxiety and neurological dysfunction after SCI in adult C57/BL6 male and female mice. After laminectomy at the 10<sup>th</sup> thoracic level, a contusive SCI was induced. The sham group received only a T10 laminectomy. Behavior testing (anxiety, motor/sensory function) was performed for 6 weeks after SCI. The spinal cord and preserved myelinated areas at the epicenter were histologically evaluated. The correlations between anxiety and motor/sensory function or histological parameters were analyzed using the Spearman correlation coefficient. Female and male mice showed significantly higher anxiety-like behaviors after SCI than before SCI. Anxiousness was significantly higher in female mice than in the male mice after SCI. There was no significant difference in motor/sensory functions and histological features between the two groups. Anxiety-like behaviors were significantly correlated with sensory function at 2 weeks after SCI in

female mice and with motor function at 2, 4, and 6 weeks after SCI in male mice.

Anxiety-like behaviors were not significantly correlated with the spinal cord area at the epicenter in female and male mice. Our results revealed that female mice became more anxious than male mice after SCI. Anxiety-like behavior after SCI may be associated with functional recovery, and improving anxiety may affect functional recovery after injury.

**Keywords:** Spinal cord injury; Anxiety; Functional recovery; Sex-related difference

## Introduction

Spinal cord injury (SCI) usually results in pain, disability, and neurological dysfunction, causing impaired mobility and loss of functional independence. Besides, after SCI, injured individuals have a Hospital Anxiety and Depression Scale score that is consistent with probable anxiety.<sup>1</sup> Some studies have performed meta-analyses or systematic reviews on anxiety or depression following SCI.<sup>2, 3</sup> Reportedly, the prevalence of anxiety based on self-reported measures is 15–32%,<sup>4</sup> and the prevalence of depression is approximately 19–26%.<sup>5</sup>

A previous study examined the relationships between inflammation, depression, and anxiety after SCI, using a rat model of SCI.<sup>6</sup> It revealed that locomotor function is not related to psychological well-being by using the sucrose preference test, open field test (OFT), and forced swim test; however, it revealed a relationship between pain and anxiety. We previously reported that SCI causes anxiety and that anxiety-model mice with phospholipase C-related catalytically inactive protein type 1 knockout show poorer motor function recovery after SCI than do wild-type mice.<sup>7</sup> Furthermore, it was reported that female adult rats have a sex-related and possibly even neuroprotective

advantage in functional recovery after thoracic contusive SCI.<sup>8</sup> Although some studies describe that SCI causes anxiety, there are no reports on sex-related differences in anxiety and functional recovery after SCI in mice. This study examined the sex-related differences in anxiety and motor/sensory recovery between adult C57BL/6J female and male mice after SCI.

## **Materials and Methods**

### *Animals*

We used 8-week-old male and female adult C57BL/6J mice weighing 18–20 g in this study. We randomly divided female and male mice into three groups (SCI group, Sham group, Naive group), each with 11 individuals. Animals were habituated to housing conditions for 5 days before experimental procedures. Estradiol cycles of all female mice were regulated. Well-trained investigators, blinded to the treatments, performed the behavioral analysis at the same time each day. All procedures involving animals were conducted according to the guidelines of the Institutional Animal Care and Research Advisory Committee of Hirosaki University.

### *Surgical and injury procedures*

Female and male mice were anesthetized with 1% isoflurane mixed with 30% oxygen. After shaving the skin, a scalpel blade was used to make a longitudinal incision along the surface of the skin on the dorsal side. After laminectomy at the 10<sup>th</sup> thoracic spinal vertebra (T10) and the exposure of the spinal cord, SCI was induced using a commercially available device (IH impactor, Precision Systems and Instrumentation,

Lexington, KY, Kentucky, USA) as described previously<sup>9</sup> by clamping the transverse processes of the vertebrae T9 and T11. This impactor uses a 2-mm impact rod tip to contuse the spinal cord, and the user determines the force of the impact, the latter being defined by the duration of the compression or “dwell-time”. For SCI groups, a force of 60 kilodynes (kdyn) was applied. After the impact, the animals were removed from the device, the muscles and fasciae were sutured with 5–0 polypropylene suture, and the skin incision was closed with autoclips. The sham group received only surgical procedure without damage, and the naive group received only anesthesia without surgical procedure.

#### *Assessment of anxiety-like behavior*

Assessment of anxiety-like behavior was measured before surgery and on days 14 (2 weeks), 28 (4 weeks), and 42 (6 weeks) after surgery.

The OFT is one of the most commonly used procedures to assess anxiety-like behavior and motor function in animal psychology and SCI studies.<sup>7, 10</sup> Spontaneous exploratory behavior was evaluated with an OFT apparatus (24 cm × 24 cm) placed in a quiet room illuminated with white light.<sup>11</sup> The floor of the apparatus was divided equally into nine

squares (8 cm × 8 cm). Each mouse was placed individually into the open field on the central square, and its spontaneous behavior was recorded by a video tracking system (Capture Star; CleverSys, Inc., Reston, Virginia, USA) for 10 min before being scored by an observer blinded to the experimental conditions.

An animal's very first exposure to the OFT can be used to assess changes in emotionality induced by exposure to a novel environment.<sup>11</sup> Rodents tend to stay in close contact with the walls of the open field apparatus in which they are placed. Mice with emotional disorders make fewer entries into the central part of the arena than do normal mice. We calculated the total distance traveled by the mouse, the average velocity, and the percentage of walking distance that fell within the central 25% of the open field using an analysis software (TopScan; CleverSys, Inc.). The percentage of distance covered in the central 25% of the field (IC-25) was calculated by dividing the walking distance covered in the central 25% area by the total walking distance.<sup>7</sup> A decrease in the IC-25 value indicates increased anxiety (Fig. 1A). In addition, the total walking distance and average acceleration were measured to evaluate motor functions.

The use of Light-Dark box test (L/D) is popular in pharmacological studies to evaluate



unconditioned anxiety responses in rodents.<sup>12</sup> The test set-up is constituted by two adjacent boxes, 24 cm × 24 cm (Light box) and 13 cm × 7 cm (Dark box). These boxes are connected at the floor level. Light intensity in the center of the Light box was set at 100 lx, whereas that in the dark box was maintained at 0 lx. For this test, mice were habituated to the Dark box for 5 min. Mice appeared at the Light box after opening the connection door and their behaviors were recorded for a 5-min period. The analyzed parameters were the percentage of time spent in the Light box using an analysis software (TopScan; CleverSys, Inc.).

The elevated plus maze (EPM) is one of the commonly used tests to measure anxiety-like behavior in mice. The test is based on the natural aversion of mice for open and elevated areas, as well as on their natural spontaneous exploratory behavior in novel environments.<sup>13</sup> There is a cruciform platform (75 cm × 75 cm) composed of two closed arms and two open arms elevated 50 cm above the ground. The two closed arms are opposite to each other with opaque walls on the sides. The remaining two arms (open arms) have no walls. All arms are connected via a central zone, allowing animals to move freely into each arm of the EPM. Their movements in the EPM were monitored

for 5 minutes with an overhead camera. The percentage of time spent in open arms ( $\text{time in open arms}/5 \text{ minutes} \times 100$ ) was analyzed using an analysis software (TopScan; CleverSys, Inc.). A decrease in the percentage of time spent in open arms indicated an increase in anxiety-like behaviors.

#### *Assessment of locomotor function*

Locomotor function was assessed throughout the 42-day recovery period with the Basso Mouse Scale (BMS), the rotarod test, and the total distance and velocity in the OFT.

The BMS is a sensitive, valid, and reliable tool for measuring locomotor function in SCI mice.<sup>14</sup> Two non-biased observers analyzed hind-limb performance using the BMS locomotor rating scale. This was evaluated on a 10-point scale (0 to 9 points) based on the paw position and trunk instability of the mouse. The BMS scores were recorded in mice by trained observers on the pre-surgery day and on days 1, 7, 14, 28, and 42 after surgery.

The rotarod (Rotarod, Ugo Basile, Varese, Italy) evaluates the balance and coordination of the mice.<sup>15, 16</sup> This test involves placing a mouse on a rotating rod to

measure the time until the mouse falls. The rotation was electronically set at a constant speed (10 rpm). We set a run time of 2 min as the maximum time. Three trials were conducted for each mouse.

#### *Assessment of sensory function*

We also evaluated cutaneous sensitivity to mechanical and thermal stimulation. In the Mechanical test, the mice were placed in plexiglass containers resting on an elevated wire mesh. Mechanical nociceptive thresholds for paw withdrawal were assessed by pressing filaments in the sole of the hind paw using a commercially available device set to automatic strength. The duration of time and the amount of pressure required to lift its hind limb by mechanical stimulation were measured (The Dynamic Plantar Aesthesiometer; Ugo Basile, Varese, Italy). The mouse was acclimatized to the testing area for 30 min before testing. Each test involved five trials on each hind paw, with at least 1 min between trials, and a randomized order of testing to minimize avoidance behaviors.<sup>17, 18</sup>

After the latency measurement, the Plantar Test Apparatus (Ugo Basile, Varese, Italy) was used to assess reactions to thermal stimulation. In this test, the mouse was placed in

a plexiglass container resting on an elevated glass surface. When the mouse was stationary and not attending to the tester or stimulus, a mobile infrared emitter (25 W) was placed under the center of the mouse's plantar hind paw from below the glass; the activation of the emitter started a timer. A photocell automatically stopped the heat source and the timer when the mouse lifted its paw. The maximum period of stimulation with heat was 20 s, at which point the heat cut off automatically to prevent tissue damage. If the mouse could not stand normally, it was held gently to assist paw placement. Each test involved five trials on each hind paw, with at least 1 min between trials, and a randomized order of testing to minimize avoidance behaviors. The latency (in s) of withdrawal from the heat source was recorded.<sup>19</sup>

### *Histological assessment*

At 6 weeks after SCI, subjects were anesthetized with isoflurane and transcardially perfused first with saline and then with 4% paraformaldehyde (0.1 M, pH 7.4). The spinal cords were removed, embedded in Optimal Cutting Temperature compound (Sakura FineTechnical Co., Ltd., Tokyo, Japan), and frozen. The frozen samples of embedded spinal cord segments were cut into 20- $\mu$ m-thick axial sections using a

cryostat (Leica CM3050 S; Germany). The spinal cords were histologically evaluated by hematoxylin-eosin (HE) staining, Luxol fast blue (LFB) staining, and immunohistochemistry.

To quantify HE and LFB stained areas, images were analyzed using the BZ-X700 software (BZ-X700, Keyence, Osaka, Japan). HE-stained images were captured at the lesion epicenter in axial sections at 40× magnification (n = 5, each). The areas of the spinal cord sections stained with HE were measured using the BZ-X700 software. To analyze the LFB-positive area, we captured regions in axial sections at the lesion epicenter at 40× magnification. The measured area in axial sections was quantified using the BZ-Analysis application (BZ-X700, Keyence, Osaka, Japan).

#### *Statistical analysis*

Behavioral-test data were analyzed using repeated-measures analysis of variance. All data are expressed as the mean  $\pm$  standard error of the mean (SEM). Analyses were performed with SPSS Version 22 (I.B.M. Corporation, Armonk, NY). Tukey's honestly significant difference test in an imbalanced one-way ANOVA model was applied to compare the means of various groups. The Spearman correlation coefficient was

determined to describe the relationships between anxiety and motor/sensory functions, as well as histological analyses. A P-value  $< 0.05$  was considered statistically significant. Any statistically significant differences between female and male mice are denoted by (\*).

## Results

### *Assessment of anxiety-like behavior*

In the anxiety tests (OFT, L/D, and EPM), there were no significant differences between female and male mice before SCI (Fig. 1B, 1D, 1F). In the OFT, the average IC-25 value significantly lower at 2, 4, and 6 weeks in the female mice ( $P = 0.02$ ,  $P = 0.01$ , and  $P = 0.02$ , respectively), as well as at 2 and 4 weeks in the male mice ( $P = 0.02$  and  $P = 0.04$ , respectively) after SCI than before SCI. Female mice of the sham and naive groups showed no significant differences in IC-25 for 6 weeks. Furthermore, no significant differences in IC-25 values were observed for the 6 postoperative weeks between female and male mice in the OFT (Fig. 1B).

In the L/D, the percentage of time spent in the Light box was significantly lower at 2 and 4 weeks in the female mice (both  $P < 0.01$ ), as well as at 4 and 6 weeks in the male mice (both  $P < 0.01$ ) after SCI than before SCI. The female mice of the SCI group spent significantly less time in the Light box than those of the sham group at 2 weeks after SCI ( $P < 0.01$ ). There was no significant difference in the percentage of time spent in the Light box between the sham and naive groups. The percentage of time in the Light

box at 2 weeks was significantly lower in the female than in the male SCI group ( $P < 0.01$ ; Fig. 1D).

In the EPM, the percentage of time spent in open arms was significantly lower at 2, 4, and 6 weeks in the female mice ( $P < 0.01$ ,  $P = 0.01$ , and  $P < 0.01$ , respectively) after SCI than before SCI. Mice of the male SCI group spent significantly lesser time in the open arms than those of the male sham group at 2 weeks after SCI ( $P = 0.02$ ). Among female mice, there were no significant differences in time between the sham and naive groups. There was also no significant difference in time spent in the open arms between female and male mice in the EPM (Fig. 1F).

#### *Assessment of locomotor function*

In the BMS, the average scores at days 1, 14, 28, and 42 after SCI were significantly lower than those before SCI in the female and male SCI groups ( $P < 0.01$ ,  $P < 0.01$ , and  $P < 0.01$ , respectively). In female and male mice, there was no significant difference in the BMS score between the sham and naive groups. Moreover, there was no significant difference in the BMS score between female and male mice after SCI (Fig. 2A).

In the rotarod test, the average riding times at 2, 4, and 6 weeks were significantly



shorter than that at pre-injury in both the female and male SCI groups (all  $P < 0.01$ ).

Furthermore, there was no significant difference in the average riding times between the sham and naive groups. Additionally, there were no significant differences in the average riding times between female and male mice after SCI (Fig. 2B).

In the OFT, the total travel distance at 2, 4, and 6 weeks after SCI was lower than that before SCI in both the female and male SCI groups (all  $P < 0.01$ ). Furthermore, the total distance at 2, 4, and 6 weeks after SCI was lower than that before SCI in female and male sham groups (female: all  $P < 0.01$ ; male:  $P < 0.01$ ,  $P = 0.03$ , and  $P < 0.01$ , respectively). The female SCI group covered a significantly shorter distance than the female sham group at 4 and 6 weeks after SCI ( $P = 0.04$  and  $P < 0.01$ , respectively). Furthermore, there was no significant difference in the total distance between female and male mice after SCI (Fig. 2C).

#### *Assessment of sensory function*

In the Mechanical test, the average reaction time at 2, 4, and 6 weeks after SCI was almost the same as the reaction time before SCI in the female and male SCI groups. Furthermore, there was no significant difference in the time between the sham and naive

groups. Additionally, there was no significant difference in the time between female and male mice after SCI (Fig. 3A).

In the Heat test, the average reaction time at 2, 4, and 6 weeks after SCI was significantly shorter than that before SCI in the male mice (SCI group) (all  $P < 0.01$ ).

Furthermore, the mean values at 2 and 6 weeks in the female SCI group were significantly higher than those in the male SCI group ( $P = 0.04$  and  $P < 0.01$ , respectively; Fig. 3B).

#### *Correlation between anxiety-like behavior and locomotor/sensory function*

We also assessed the correlation between IC-25 values and locomotor/sensory functions on before and 2, 4, and 6 weeks after SCI. The IC-25 values were significantly correlated with those in the Heat test at 2 weeks after SCI among female mice, those in the BMS at 2, 4, and 6 weeks after SCI among male mice, and those in the rotarod test at 2 and 4 weeks after SCI among male mice (Tables 1, 2). Single correlation analyses revealed significant positive correlations between anxiety and sensory functions in female mice, as well as between anxiety and motor functions in male mice.

#### *Histological assessment and correlation between anxiety-like behavior and spinal cord*

*area*

Atrophic changes and demyelination of the injured spinal cord were examined 6 weeks after SCI by HE and LFB staining (Fig. 4A-H). There was no significant difference in the transverse area of the spinal cord at the lesion epicenter between female and male SCI mice (Fig. 4I, 4J). Anxiety-like behaviors were not significantly correlated with spinal cord areas (Tables 1, 2).

## Discussion

To our knowledge, this is the first study to investigate sex-related differences in anxiety and functional recovery after SCI in mice. Our results revealed that, after the procedure (SCI/sham), both female and male mice of the SCI group exhibited higher anxiety-like behaviors than those of the sham group did. However, 2 weeks after the injury, L/D revealed that female mice became more anxious than male mice did, whereas it revealed that male mice became more hypersensitive than female mice. In female mice, the anxiety-like behavior was correlated with mechanical hypersensitivity in the subacute phase after SCI, whereas it was correlated with the lack of motor function recovery in the subacute phase after SCI in male mice.

In this study, we assessed anxiety-like behaviors of mice by using the OFT, EPM, and L/D. All of these tests revealed anxiety-like behavior in mice after SCI; among them the OFT was the most conclusive test. In previous reports, anxiety was evaluated in rodents after SCI using the OFT<sup>7</sup> and EPM.<sup>6, 20, 21</sup> These studies used the OFT to compare anxiety-like behavior after SCI to that before SCI<sup>6, 7</sup> and used EPM to assess anxiety-like behaviors in a rat model of SCI.<sup>6, 20, 21</sup> In the OFT, mice were evaluated for

anxiety-like behavior based on search behavior in a novel environment. However, search behavior in a novel environment decreases when rodents get used to the environment; therefore, multiple assessments are important for the evaluation of anxiety-like behavior in mice. Accordingly, our multiple assessments using the OFT, EPM, and LD can be useful for the evaluation of anxiety-like behavior in mice after SCI.

Anxiety after spinal cord injury is likely to be related to inflammation. SCI results in increased inflammation both peripherally and centrally in male rats.<sup>6</sup> do Espirito Santo et al. suggested that SCI by clip-compression in female rats promotes a neuropsychiatric-like profile associated with an imbalance in the production/release of pro- and anti-inflammatory cytokines.<sup>20</sup> Furthermore, female mice with an abnormality of GABA<sub>A</sub>-receptor function showed increased inflammation after SCI, and the increased inflammation resulted in anxiety and motor functional disability.<sup>7</sup> Similarly, the current study showed that both male and female mice became anxious after SCI. Furthermore, female mice became significantly more anxious than male mice did in the SCI groups. Female sex hormones such as estrogens and progesterone affect emotions

and cognition, contributing to sex differences in behavior.<sup>22</sup> A previous study in rats demonstrated that age, sex, and gonadal hormones differently influence anxiety- and depression-related behaviors during puberty in mice.<sup>23</sup> Therefore, our findings indicate that sex hormones such as estrogens and progesterone affected the difference in anxiety-like behavior between female and male mice.

In this study, there was no significant difference in motor function between the sexes. It has been reported that adult female rats have a sex-related and possibly even neuroprotective advantage in functional recovery after thoracic, contusive SCI.<sup>8</sup> However, it has also been reported that there is no significant difference in locomotor recovery between male and female mice, which were assessed under open field and treadmill conditions.<sup>24</sup> Furthermore, Swartz et al.<sup>25</sup> found a similar improvement in Basso-Beattie-Bresnahan scores of adult male and female rats. Our results corroborate the result of this study, that is, there are no sex differences in locomotor function in mice after SCI. Datto et al. showed that female rats with moderate thoracic spinal cord contusion injury induced by dropping a 10.0-g rod from a height of 12.5 mm showed improvement in locomotor function and that the functional improvement is because of a

possible neuroprotective effect.<sup>8</sup> In their histological analysis at 13 weeks post-SCI, area of the rat thoracic spinal cord at the injury epicenter was significantly larger in female rats than in male rats. By contrast, our study revealed no significant difference in the histological analysis between female and male mice after SCI. Thus, we could not identify sex differences in locomotor function. The difference in results between our histological analysis and their histological analysis may have been because of the severity of the injury, the timing of the assessment, and differences between mice and rats.

In the Heat test, but not mechanical test, male mice were more hypersensitive to thermal stimuli than male sham group after SCI. On the contrary, female mice did not become hypersensitive after SCI. It was reported that female rats develop hypersensitivity more rapidly and to a greater extent than male rats do after SCI<sup>26</sup>. Besides, it was shown that estrogen protects Schwann cells and has a neuroprotective effect on the peripheral nervous system.<sup>27</sup> Therefore, we suggest that sensory functions recovered earlier in female mice than in male mice after SCI. The protective effect of estrogen on Schwann cells was more effective on sensory functions of peripheral

nerves; therefore, the discrepancy between motor and sensory recovery may have occurred.

Anxiety-like behavior after SCI was correlated with mechanical hypersensitivity in female mice, as well as with the loss of motor function in male mice. There was no significant difference in anxiety like-behavior between the sham and naive groups; this suggests that anxiety-like behavior was unrelated to stress caused by operation and anesthesia. There have been a few reports describing the correlation between anxiety and motor function, and previous reports demonstrated a positive correlation between anxiety-like behavior and hypersensitivity in female<sup>21</sup> and male<sup>6, 28</sup> rats. Fujita showed that in female SCI model mice, the IC-25 value is correlated with motor-sensory functions.<sup>7</sup> The reason may be that pain hypersensitivity in female mice is affected by cells of the adaptive immune system.<sup>29</sup> Our results showed that there was a correlation between anxiety-like behavior and motor function in male mice, as well as anxiety-like behavior and sensory function in female mice.

There are some limitations to this study that should be acknowledged. First, all mice did not perform the behavioral tests at the same time, and their performance may have



been affected by environmental factors such as temperature or humidity. A parallel-group comparison study may reduce bias when observing differences between mice. Second, hormonal imbalances due to the female reproductive cycle may affect anxiety-like behavior, but we did not determine whether sex hormone levels were constant. Third, although anxiety was examined using established evaluation methods, there have been few studies that measured anxiety in mice after SCI. Thus, the correct evaluation of anxiety in mice may have been altered by the SCI-induced paralysis. Although this study had some limitations, it will contribute to the elucidation of the relationship between anxiety and locomotor dysfunction after SCI. Prescribing anxiolytics for patients with SCI may help improve the affected motor/sensory function, thereby improving the quality of life of patients affected by SCI.

## **Conclusion**

Our results revealed that both female and male mice after SCI showed increased anxiety-like behaviors with female mice becoming more anxious than male mice and male mice becoming more hypersensitive than female mice. Anxiety-like behavior was correlated with mechanical hypersensitivity in a subacute phase after SCI in female

mice, whereas it was correlated with the loss of motor function in a subacute phase after SCI in male mice.

## **Acknowledgements**

We thank the members of the Graduate School of Medicine, Department of Orthopedic surgery, for their excellent guidance and advice. This study was funded by the Karoji Memorial Fund for Medical Research at Hirosaki University, a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (17K10917), and a Hirosaki University Grant for Distinguished Researchers (FY2017-2018). We would like to thank Editage for English language editing.

## **Author Disclosure Statement**

The authors have no conflicts of interest directly relevant to the content of this article.

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## Figure Legends

**Fig. 1.** Assessment of mice in anxiety tests. **(A)** Representative images displaying traces of sham-operated and SCI mice in the open field test (OFT). The squares indicate the IC-25 areas, used to quantify anxiety in mice. **(B)** Comparison of the IC-25 values among the SCI (SCI), sham (Sh), and naive (N) groups at pre-surgery and days 14, 28, and 42 after surgery. The IC-25 values of the SCI groups were decreased after the injury, whereas there was no significant change in the sham and naive groups. The IC-25 values revealed lower anxiety in the SCI groups compared to the sham and naive groups at 2, 4, and 6 weeks after SCI. Values represent the mean  $\pm$  SEM ( $n = 11$ , each). \* $P < 0.05$  female versus male, ANOVA. # $P < 0.05$  versus pre-injury, ANOVA. **(C)** Representative images of sham and SCI model mice in the Light/Dark test (L/D). The images display the Light box. **(D)** Comparison of the percentages of time spent in the Light box among the SCI (SCI), sham (Sh), and naive (N) groups at pre-surgery and days 14, 28, and 42 after surgery. The time spent in the Light box revealed lower anxiety in the SCI group than in the sham and naive groups at 2 weeks after SCI. \* $P < 0.05$  female versus male, ANOVA. # $P < 0.05$  versus pre-injury, ANOVA. **(E)**

Representative images of sham and SCI model mice in the elevated plus maze test (EPM). There are two closed arms (opposing each other) and two open arms (opposing each other) without walls. (F) Comparison of the percentages of time spent in the open arms among the SCI (SCI), sham (Sh), naive (N) groups at pre-surgery and days 14, 28, and 42 after surgery. Although the time in the open arms decreased after injury in the SCI group, there were no significant changes in the sham and naive groups. The time spent in the open arms by mice of the SCI group was lower than that in the sham group at 6 weeks after SCI. \*P < 0.05 female versus male, ANOVA. #P < 0.05 versus pre-injury, ANOVA.

**Fig. 2.** Assessment of motor functions. (A) Comparison of BMS scores among the three groups (SCI, sham [Sh], and naive [N]) at pre-surgery and days 14, 28, and 42 after surgery. The BMS scores in the SCI group were lower than those in the sham and naive groups after SCI. The BMS scores in the SCI group improved gradually after SCI. \*P < 0.05 female versus male, ANOVA. †P < 0.05 versus naive, ANOVA. (B) Comparison of the average riding times in the rotarod test among the three groups (SCI, sham, and

naive) at pre-surgery and days 14, 28, and 42 after surgery. The time in the SCI group was significantly lower than those in the sham and naive groups after SCI. The time of the rotarod test in the SCI group showed no change after SCI. \* $P < 0.05$  female versus male, ANOVA. † $P < 0.05$  versus naive, ANOVA. (C) Comparison of the average activity in the open field test (OFT) among the three groups (SCI, sham, and naive) at pre-surgery and days 14, 28, and 42 after surgery. There was no significant difference in the distance between females and males in the SCI group. \* $P < 0.05$  female versus male, ANOVA. † $P < 0.05$  versus naive, ANOVA.

**Fig. 3.** Assessment of sensory functions. (A) Comparison of the reaction time in the Dynamic Plantar test among the three groups (SCI, sham [Sh], and naive [N]) at pre-surgery and days 14, 28, and 42 after surgery. The time in the Mechanical test showed in the SCI group no significant change after SCI. \* $P < 0.05$  female versus male, ANOVA. † $P < 0.05$  versus naive, ANOVA. (B) Comparison of the reaction time in the Plantar test among the three groups (SCI, sham [Sh], and naive [N]) at pre-surgery and days 14, 28, and 42 after surgery. The reaction time of heat test in the SCI group was

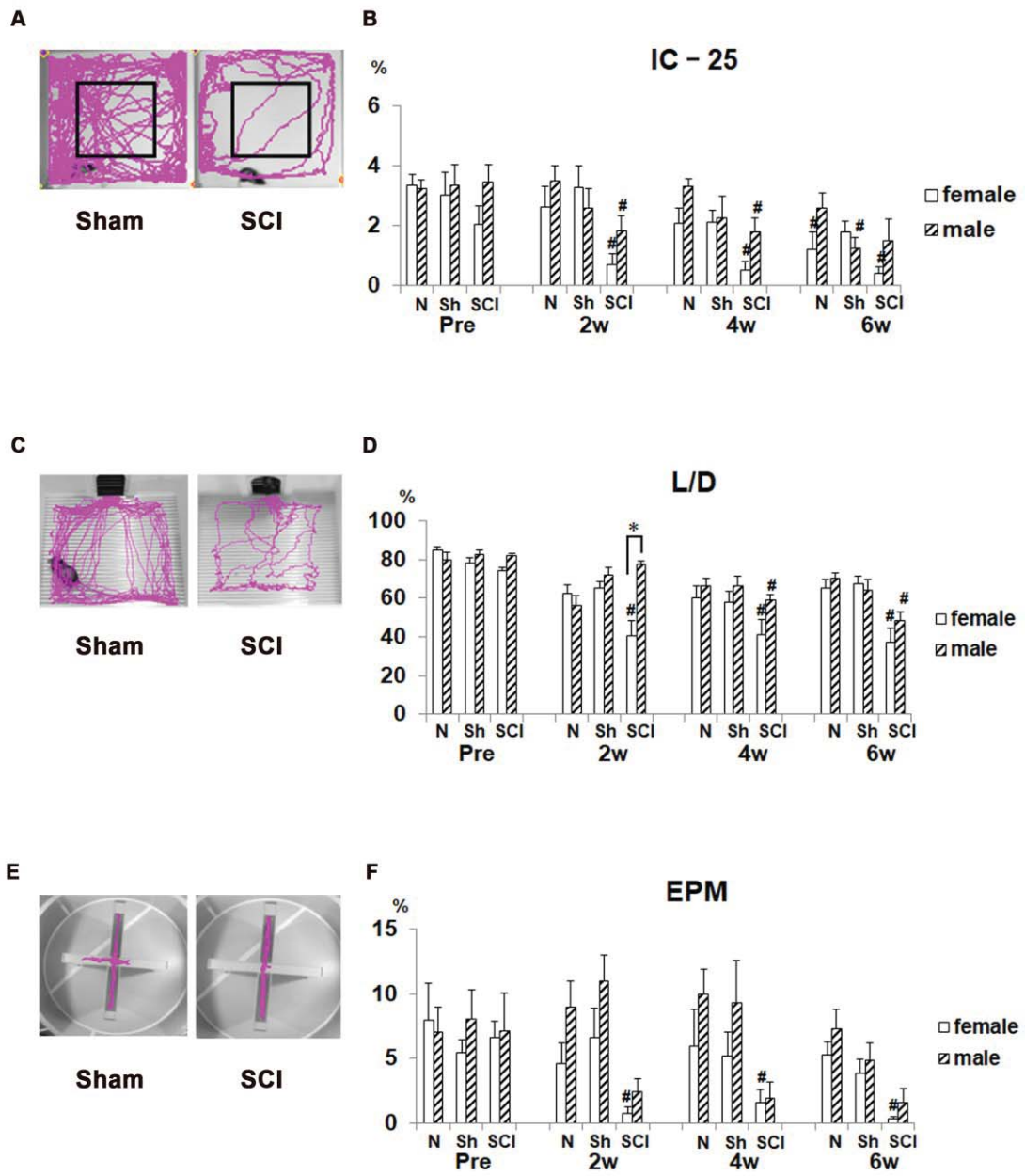
significantly decreased after SCI. \* $P < 0.05$  female versus male, ANOVA. † $P < 0.05$  versus naive, ANOVA.

**Fig. 4.** Spinal-cord atrophy and demyelination 6 weeks after injury. Representative HE-stained (**A, B, E, F**) and LFB-stained (**C, D, G, H**) axial spinal-cord sections at the 10<sup>th</sup> level of male and female sham-operated and SCI model mice. Scale bars: 100  $\mu$ m.

(**I**) Bar graph showing that the transverse spinal-cord area at the lesion epicenter was no significant difference between female and male SCI mice ( $n = 5$  each). \* $P < 0.05$

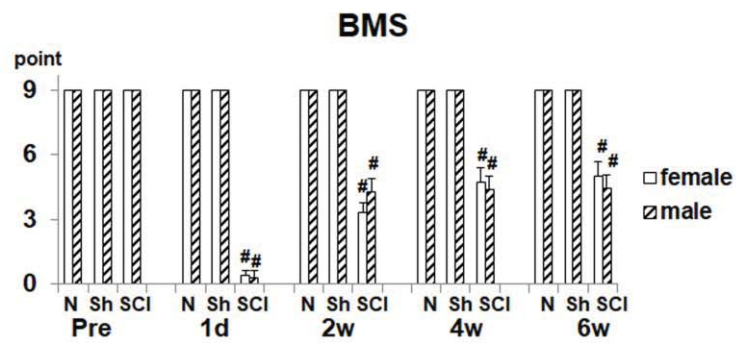
(**J**) The area of LFB staining at the lesion epicenter was no significant difference between female and male SCI mice ( $n = 5$  each). \* $P < 0.05$

**Figure 1**

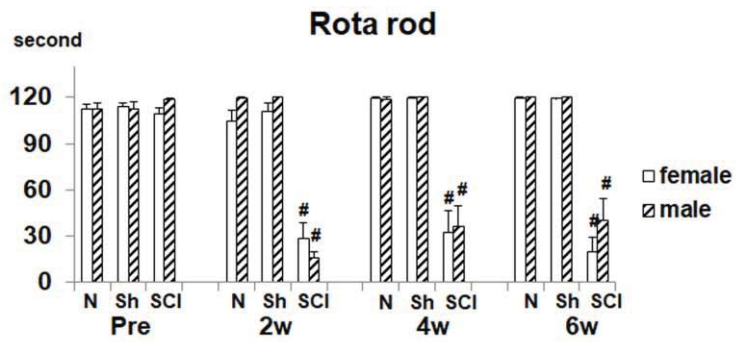


**Figure 2**

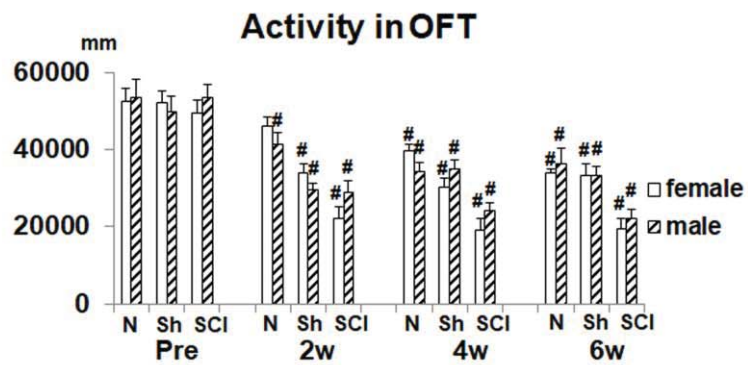
**A**



**B**



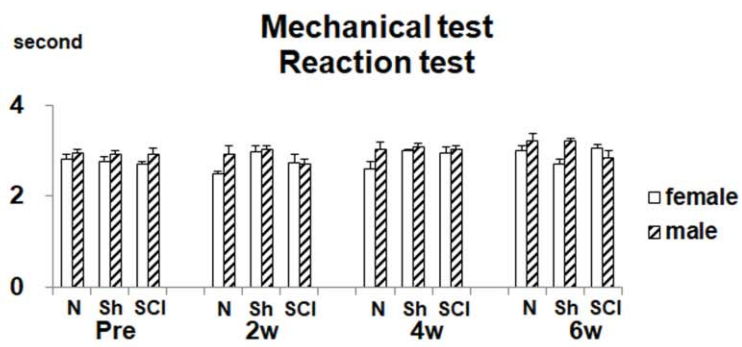
**C**





**Figure 3**

**A**



**B**

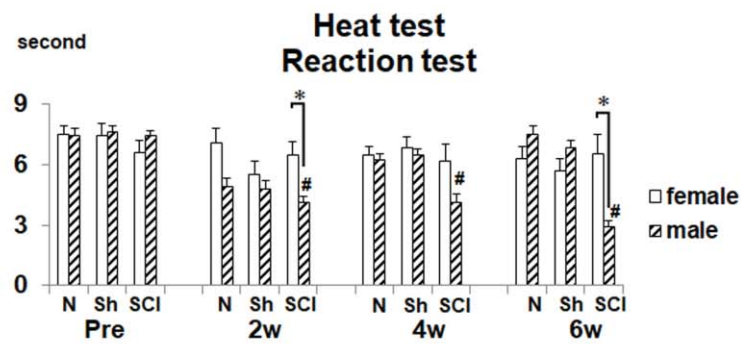


Figure 4

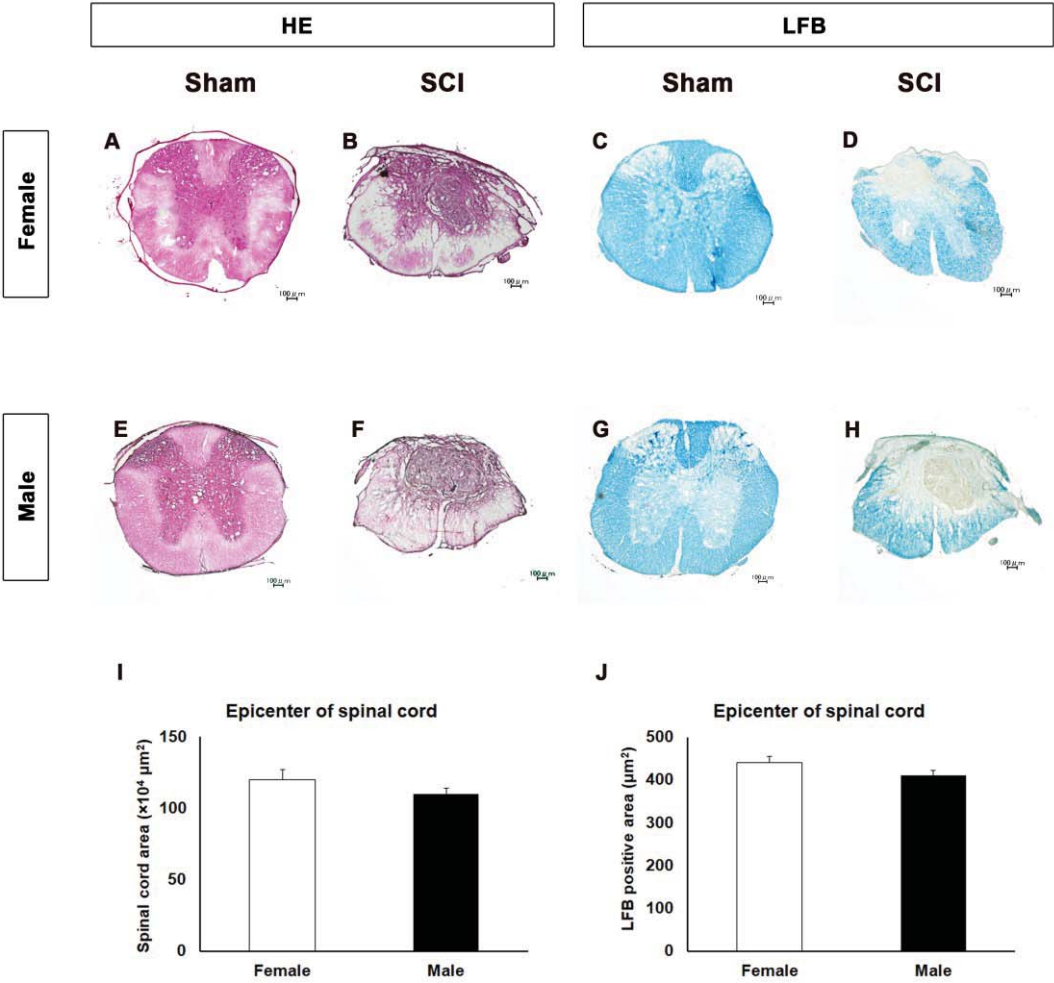


Table 1. Correlation between IC-25 values and locomotor/sensory functions in female mice after SCI

		Locomotor function		Sensory function	
		BMS	Rota	Mechanical	Heat
Pre	R(P)		-0.44 (0.21)	0.32 (0.36)	-0.68 (0.03) <sup>†</sup>
2 weeks	R(P)	0.29 (0.42)	0.42 (0.23)	-0.30 (0.40)	0.67 (0.03) <sup>†</sup>
4 weeks	R(P)	0.26 (0.46)	0.51 (0.13)	-0.16 (0.66)	-0.19 (0.60)
6 weeks	R(P)	-0.19 (0.59)	0.01 (0.98)	0.21 (0.55)	-0.21 (0.55)

<sup>†</sup>A significant correlation with an adjusted P-value < 0.05.

R, correlation coefficient (R > 0 means a perfect positive correlation and R < 0 means a perfect negative correlation); IC-25, percentage of distance spent in the center 25% of the open field; SCI, spinal cord injury; BMS, Basso Mouse Scale.

Table 2. Correlation between IC-25 values and locomotor/sensory functions in male mice after SCI

		Locomotor function		Sensory function	
		BMS	Rota	Mechanical	Heat
Pre	R(P)		-0.09 (0.77)	0.10 (0.75)	0.15 (0.65)
2 weeks	R(P)	0.84 (< 0.01) <sup>†</sup>	0.62 (0.04) <sup>†</sup>	-0.29 (0.38)	0.20 (0.54)
4 weeks	R(P)	0.82 (< 0.01) <sup>†</sup>	0.78 (< 0.01) <sup>†</sup>	0.37 (0.25)	0.25 (0.44)
6 weeks	R(P)	0.67 (0.02) <sup>†</sup>	0.40 (0.21)	0.31 (0.35)	0.20 (0.54)

<sup>†</sup>A significant correlation with an adjusted P-value < 0.05.

R, correlation coefficient (R > 0 means a perfect positive correlation and R < 0 means a perfect negative correlation); IC-25, percentage of distance spent in the center 25% of the open field; SCI, spinal cord injury; BMS, Basso Mouse Scale.

Table 3. Correlation between IC-25 values and spinal cord areas (epicenter) after SCI

		Female	Male
Pre	R(P)	-0.30 (0.62)	-0.10 (0.87)
2 weeks	R(P)	-0.11 (0.85)	-0.30 (0.61)
4 weeks	R(P)	-0.11 (0.85)	-0.46 (0.43)
6 weeks	R(P)	-0.44 (0.45)	-0.10 (0.86)

R, correlation coefficient (R > 0 means a perfect positive correlation and R < 0 means a perfect negative correlation); IC-25, percentage of distance spent in the center 25% of the open field; SCI, spinal cord injury.